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The Ca^{2+}-dependent Binding of Calmodulin to an N-terminal Motif of the Heterotrimeric G Protein β Subunit*

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Mingyao Liu, Bo Yu, Osamu Nakanishi, Thomas Wieland, and Melvin Simon‡

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

Ca^{2+} ion concentration changes are critical events in signal transduction. The Ca^{2+}-dependent interactions of calmodulin (CaM) with its target proteins play an essential role in a variety of cellular functions. In this study, we investigated the interactions of G protein $\beta\gamma$ subunits with CaM. We found that CaM binds to known $\beta\gamma$ subunits and these interactions are Ca^{2+}-dependent. The CaM-binding domain in G$\beta\gamma$ subunits is identified as Gβ residues 40–63. Peptides derived from the Gβ protein not only produce a Ca^{2+}-dependent gel mobility shifting of CaM but also inhibit the CaM-mediated activation of CaM kinase II. Specific amino acid residues critical for the binding of G$\beta\gamma$ to CaM were also identified. We then investigated the potential function of these interactions and showed that binding of CaM to G$\beta\gamma$ inhibits the pertussis toxin-catalyzed ADP-ribosylation of Gαo subunits, presumably by inhibiting heterotrimer formation. Furthermore, we demonstrated that interaction with CaM has little effect on the activation of phospholipase C-β2 by G$\beta\gamma$ subunits, supporting the notion that different domains of G$\beta\gamma$ are responsible for the interactions of different effectors. These findings shed light on the molecular basis for the interactions of G$\beta\gamma$ with Ca^{2+}-CaM and point to the potential physiological significance of these interactions in cellular functions.

In response to external stimuli or changes in ligand concentration, the activated seven-transmembrane receptors of the cell interact with heterotrimeric G proteins. These in turn activate or inhibit a variety of intracellular proteins through either the Gα subunits with GTP bound or the free G$\beta\gamma$ subunits or both, leading to the generation of second-messenger molecules and changes in the patterns of cellular metabolic activity and growth. Whereas direct interaction of G protein α subunits with effectors has been known for a long time, recently it has been demonstrated that G$\beta\gamma$ subunits also play critical roles in effector activation, in modulating the interaction among various G protein pathways, and in regulating cellular functions. For example, the G$\beta\gamma$ dimers have been shown to participate in interactions with adenylyl cyclases (1), phospholipases (2, 3), phosducin (4), β-adrenergic receptor kinases (5–7), Bruton tyrosine kinase (8), inositol trisphosphate kinases (9, 10), mitogen-activated protein kinase (11), and a number of ion channels (12–16). The activation of phospholipase C-β2 and β3 by G$\beta\gamma$ has been suggested to account for the PTX[1]-sensitive increase in intracellular Ca^{2+} in response to a number of chemoattractants (17, 18) and other ligands, thus regulating intracellular Ca^{2+} concentration.

Calmodulin (CaM) has been known to act as an intracellular calcium sensor protein. When the intracellular Ca^{2+} concentration increases, CaM can bind up to four Ca^{2+} ions, changing its conformation and regulating cellular functions such as activation or inhibition of a large number of enzymes (19, 20), ion channels (21), and receptors (22). These Ca^{2+}-dependent interactions of CaM with its target proteins have played an important role in intracellular Ca^{2+} signaling and in various cellular functions including cell growth and differentiation. There are reports demonstrating that G protein $\beta\gamma$ complex can bind to CaM when passed through the CaM-agarose column (23, 24); however, the precise nature of the interaction is not clear.

In this report, we investigated the interaction of Ca^{2+}-CaM with G$\beta\gamma$ subunits and the potential physiological significance of this interaction. We found that CaM not only binds to G$\beta\gamma$ subunits purified from brain but also to the most diverse Gβ_5Lγ complex from retina. We then identified and characterized the CaM-binding domain of the Gβ subunit by using synthetic peptides and site-specific mutation. Furthermore, we showed that binding of CaM to $\beta\gamma$ inhibits the $\beta\gamma$-dependent PTX-catalyzed ADP-ribosylation of Gα subunits. Using both brain G$\beta\gamma$ subunits and the CaM-binding peptide derived from the β subunit, we demonstrated that the Ca^{2+}-CaM-dependent activation of CaM kinase II could be inhibited at a molar ratio of 1 (peptide/CaM). These studies provide insight into the molecular basis for the interactions of $\beta\gamma$ with Ca^{2+}-CaM and the potential functions of these interactions in modulating cellular signaling and other functions.

EXPERIMENTAL PROCEDURES

Materials

Synthetic peptides were obtained from the Peptide Synthesis Facility (Beckman Institute, California Institute of Technology). Peptides were purified by high performance liquid chromatography and verified by mass spectrometry. Bovine brain CaM was purchased from Calbiochem. Phosphatidylethanolamine and phosphatidylinositol-4,5-diphosphate ($PtdInsP_2$) were purchased from Avanti Polar Lipids (Alabaster, AL) and Boehringer Manheim, respectively. [^{3}H]$PtdInsP_2$, *myo*-[2-^{3}H]inositol, and [^{32}P]NAD were obtained from DuPont NEN. Pertussis toxin was purchased from List Biological Laboratories Inc.

CaM Binding Assay of G Protein β5Lγ Subunits

Bleached bovine rod outer segment membranes were prepared from bovine retina as described (25). Transducin was removed from the membranes by 6 × hypotonic elution with 100 μM GTPγS in the presence of 3 mM $MgCl_2$ (26). The remaining membranes were solubilized for 12 h at 4 °C in 20 ml of a buffer containing 50 mM Hepes, pH 8.0, 100

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‡ To whom correspondence should be addressed: Division of Biology, 147-75, California Institute of Technology, Pasadena, CA 91125. Tel.: 818-395-3944; Fax: 818-796-7066; E-mail: simonm@starbase1.caltech.edu.

[1] The abbreviations used are: PTX, pertussis toxin; CaM, calmodulin; $PtdInsP_2$, phosphatidylinositol-4,5-diphosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; PLC-β2, phospholipase C-β2.

mM NaCl, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 1% (by mass) sodium cholate. Unsolubilized material was pelleted for 40 min at 100,000 × *g*, and the supernatant was diluted with 30 ml of a buffer containing 50 mM Hepes, pH 8.0, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 1.67 mM $CaCl_2$ (final concentration: 1 mM $CaCl_2$, 0.4% sodium cholate). The diluted supernatant was then applied at a flow rate of 0.3 ml/min to a CaM-Sepharose column (10-ml bed volume, Pharmacia Biotech Inc.) equilibrated with the above described buffer. The column was washed with 50 ml of the above buffer. Proteins bound to the CaM-Sepharose column were then isocratically eluted with a buffer containing 50 mM Hepes, pH 8.0, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 mM EDTA, and 0.4% sodium cholate. Fractions of 600 μl were collected. 20 μl of the indicated fractions were separated by 10% SDS-PAGE and immunoblotted using the $G\beta_5$-specific antiserum CT215 as described (27).

Gel Mobility Shifting and Gel Overlay Assays

High affinity binding of G protein β peptides to CaM was demonstrated by the gel mobility shifts of CaM in 12.5% nondenaturing polyacrylamide gels in the presence or absence of 4 M urea. Different concentrations of Gβ peptide were incubated with 1 μM CaM at room temperature for 30 min. To determine the Ca^{2+} dependence and binding stoichiometry, gels were run in the presence of either 0.5 mM $CaCl_2$ or 2 mM EGTA. Proteins were visualized by Coomassie Brilliant Blue staining.

Fluorescence Measurements and Determination of Dissociation Constants

Fluorescence emission spectra were obtained using SLM 4800 spectrofluorimeter. Excitation was at 295 nm. Excitation and emission band-passes were both 10 nm. Total fluorescence was determined by integration of emission spectra. The fluorescence titration data was used to determine the dissociation constant as described previously (28). By plotting the fraction of bound peptide as a function of free CaM concentrations, we obtained the peptide-CaM titration curve. The dissociation constant for the peptide-CaM binding was obtained from the curve fitting.

Site-specific Mutagenesis

Mutations in the putative CaM-binding site of $G\beta_1$ were generated by polymerase chain reaction with the high fidelity DNA polymerase, *Pfu* (Stratagene). The mutations were confirmed by DNA sequencing done in the DNA sequencing facility at Caltech. The β constructs were subcloned into pCDNA3.1 vector (Invitrogen, San Diego, CA).

In Vitro Translation and Binding Assays

Transcription and translation were carried out using a rabbit reticulocyte lysate system from Promega at 30 °C for 90 min with amino acid mixtures without methionine. 5 μCi of [^{35}S]methionine (>1000 Ci/mmol, DuPont NEN) was added to the reaction mixture to monitor the synthesis of new proteins. 1–2 μg of cDNAs were used in each translation. 2.5 μl aliquots of the translation mixture were separated on 12% SDS-PAGE. The newly synthesized proteins were identified by autoradiography. After *in vitro* translation, the protein mixture was incubated with CaM-agarose beads in the presence of Ca^{2+} for 30 min at room temperature. Then the CaM-agarose beads were washed extensively to get rid of nonspecific binding. Proteins bound to the beads were eluted by a buffer containing 10 mM EGTA. The eluted Gβ proteins were separated by SDS-PAGE and visualized by autoradiography.

ADP-ribosylation of Gαo Subunits by PTX

Pertussis toxin-catalyzed ADP-ribosylation of Gαo was performed as described previously (29). Briefly, 0.1 μg of recombinant Gαo was mixed with 0.1 μg of purified rat brain βγ subunits in the absence or presence of Ca^{2+}-CaM and incubated for 10 min at room temperature before the addition of the reaction mixture (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 μM ^{32}P-labeled NAD (20,000 cpm/pmol), and 10 μg/ml pertussis toxin). Reactions were incubated at room temperature for 30 min and terminated by adding 5 × SDS-PAGE sample buffer. Samples were resolved on SDS-polyacrylamide gels and stained with Coomassie Blue. Labeling of proteins was detected by autoradiography.

Enzymatic Activity Assays of CaM Kinase II

The Ca^{2+}-CaM-dependent activity of CaM kinase II was assayed essentially as described previously (30). Briefly, different concentrations of Gβ peptide were preincubated with Ca^{2+}-CaM for 30 min at room temperature. The phosphorylation reactions contained 50 mM Hepes buffer, pH 7.5, 10 mM magnesium acetate, 1 mM $CaCl_2$, 0.1 mM [γ-^{32}P]ATP, 1 μM CaM, 5–10 μg of CaM kinase II peptide substrate (Chemicon International, Inc., Temecula, CA), and purified rat brain CaM kinase II (Calbiochem). All assays were initiated by the addition of kinase.

COS-7 Cell Transfection and Phosphoinositol Phospholipase C Activity Assays

In Vivo Transfection Assay—cDNAs encoding PLC-β2, Gβγ, and CaM were cotransfected into COS-7 cells with LipofectAMINE (Life Technologies, Inc.). Then the cells were labeled with 10 μCi/ml *myo*-[2-^{3}H]inositol the following day. 48 h after transfection, the activity of PLC-β2 was assayed by determining the levels of inositol phosphates as described previously (31, 37).

In Vitro Reconstitution Assay of PLC-β2 Activity— Phospholipid vesicles containing 50 μM [^{3}H]PtdInsP_2 and 500 μM phophatidylethanolamine were prepared by mixing with chloroform solution, drying under a stream of N_2, then sonicating with 88 mM Hepes buffer, pH 7.5, and 18 mM LiCl. Assays were performed in a 70-μl reaction mixture containing 20 ng of PLC-β2, 1.7 μM Gβγ from bovine retina, 50 μM $CaCl_2$, 10 mM LiCl, and phospholipid vesicles. The reaction mixtures were incubated at 30 °C for 10 min in the presence of different concentrations of CaM. The reactions were stopped by adding 0.35 ml of chloroform/methanol/HCl (500:500:3). The released Ins-1,4,5-P_3 was extracted by adding 0.1 ml of 1 M HCl with vigorous vortexing. The aqueous phase separated after centrifugation was subjected to scintillation counting.

RESULTS

Binding of Gβγ Subunits to Ca^{2+}-CaM—To demonstrate the direct interaction of Gβγ subunits with CaM, purified bovine brain βγ subunits were incubated with CaM-agarose gel in the presence of 1 mM $CaCl_2$. After extensively washing with Ca^{2+}-containing buffer, the bound βγ subunits were eluted by buffer containing EGTA. As shown in Fig. 1*A*, brain βγ binds to CaM, and this binding is Ca^{2+}-dependent. Direct binding was also observed in a gel overlay assay with ^{125}I-labeled CaM (data not shown). There are five different G protein β subunits that have been characterized in mammalian systems. Among them, $G\beta_5$ is the most diverse form and is expressed predominately in neuronal cells (27). A splice variant of $G\beta_5$, β_5L, is found only in rod outer segment membrane. To examine whether CaM binding is a common feature in different βγ subunits, $G\beta_5L\gamma$ extracts were obtained from bovine retina and passed through a CaM-agarose fast protein liquid chromatography column in the presence of Ca^{2+}. Proteins bound to the CaM-agarose column were eluted with buffer containing 10 mM EDTA and detected by specific antibodies against the $G\beta_5$ subunit. Like other βγ subunits, $\beta_5L\gamma$ also binds to Ca^{2+}-CaM (Fig. 1*B*), suggesting that the CaM-binding domain is conserved in all Gβγ subunits.

Identification of a CaM-binding Domain of Gβγ Subunits—To identify the CaM-binding domain on Gβγ subunits, the amino acid sequences of βγ were compared with those of known high affinity CaM-binding proteins. We found that the N terminus of all Gβ subunits contains a putative CaM-binding domain that exhibits the characteristics typical of CaM-binding peptides (32). Fig. 2 shows the alignment of the N-terminal domain sequences of Gβ subunits with well known CaM-binding domains of rat olfactory cyclic nucleotide-gated channel (*RAT OCNC*), skeletal muscle myosin light chain kinase (*SK-MLCK*), Ca^{2+} pump, calcineurin, Ras-like GTPase Kir/Gem, murine inducible nitric oxide synthase (*iNOS*), and CaM kinase II. To demonstrate that the small domain from amino acid residues Val-40–Trp-63 is indeed the CaM-binding domain of Gβ subunits, synthetic peptides representing the putative CaM-binding domain were prepared and tested for their abilities to bind CaM in the presence of Ca^{2+} using both gel mobility shifting assay and tryptophan fluorescence assays.

Binding of peptides to CaM was first assayed by nondenaturing polyacrylamide gel shifting assay. Depending on the

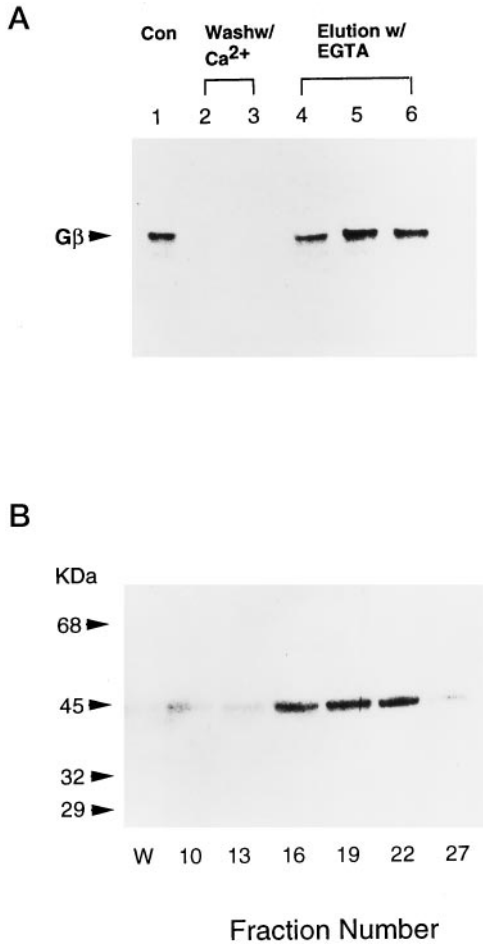

FIG. 1. **Binding of G protein βγ subunits to Ca^{2+}-CaM.** *A*, interaction of rat brain Gβγ subunits with Ca^{2+}-CaM. Purified rat brain βγ subunits were incubated with CaM-agarose in the presence of 1 mM $CaCl_2$ for 30 min at room temperature. Then, the CaM-agarose complexes were washed extensively with phosphate-buffered saline containing $CaCl_2$. Proteins bound to CaM-agarose were eluted with buffer containing EGTA. Eluted Gβ protein was visualized by Western blot using specific antibodies against Gβ subunits. *Con*, control. *B*, binding of $G\beta_5L\gamma$ subunits to Ca^{2+}-CaM. $G\beta_5L\gamma$ extracts were obtained from bovine retina and were then applied to a CaM-Sepharose fast protein liquid chromatography column (Pharmacia) equilibrated with Ca^{2+}-containing buffer. Proteins bound to the CaM-Sepharose column were then eluted with an EDTA-containing buffer. 20 μl of the indicated fractions were separated by 10% SDS-PAGE and immunoblotted using the $G\beta_5$-specific antiserum CT215.

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Gβ subunit    VGRIQMRTRRTLRRHLAKIYAMH-WG
RAT OCNC           RRGRGGFQRIVRLVGVIRDWANKNKF
SK-MLCK M13           KRRWKKNFIAVSAANRFKKISSSGAL
Ca2+-Pump             QILWFRGLNRIQTQIRVVNAFRSS
Calcineurin         ARKEVIRWKIRAIGKMARVSFVL
Murin iNOS        RPRRREIRFRVLVKVVFFASMLM
Kir/Gem             KARRFWGKIVAKNNKNMAFKLKSKS
CaM Kinase II   LKKFNARRKLKGAILTTMLATRNFS
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FIG. 2. **Alignment of the putative CaM-binding domain in Gβ subunit with known CaM-binding proteins.** The putative CaM-binding sequences of Gβ subunits are compared with the known CaM-binding domains of rat olfactory cyclic nucleotide-gated channel (*OCNC*) (21), skeletal muscle myosin light chain kinase (*skMLCK*) (40), plasma membrane Ca^{2+}-ATPase (*Ca^{2+} pump*) (41), calcineurin (42), Ras-like GTPase Kir/Gem (43), murine-inducible nitric oxide synthase (*Murin iNOS* residues 501–532) (44), and CaM kinase II (45). The conserved hydrophobic residues are *boxed* and may play an important role in the interaction with CaM.

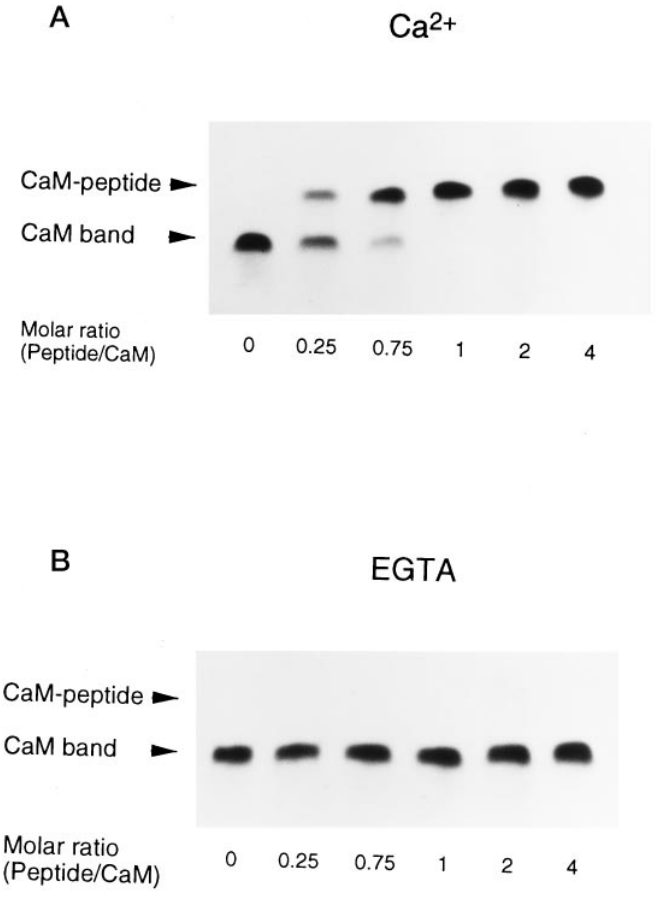

FIG. 3. **Gel mobility shifting assay of CaM by synthetic peptide.** CaM (10 μg) was incubated with the indicated amount of peptide derived from Gβ subunit in the presence of 1 mM $CaCl_2$ (*A*) or 2 mM EGTA (*B*). Samples were then separated on 12% nondenaturing polyacrylamide gels containing either $CaCl_2$ or EGTA. The relative mobility of CaM and CaM-peptide complex was visualized by Coomassie Blue staining. The molar ratios of peptide to CaM are shown as indicated. The results are representative of three separated experiments.

charges and hydrophobicity of the peptide, high affinity binding of the peptide to CaM was detected as a complex band with increased or decreased mobility compared with the unbound CaM band. In the presence of Ca^{2+}, the mobility of CaM was decreased by the peptide corresponding to Gβ residues 40–63 (Fig. 3*A*). Several ratios of the peptide to CaM were used. In the absence of peptide, there is a single, fast-moving band reflecting pure Ca^{2+}-CaM (Fig. 3*A*, CaM alone). At ratios of 0.25 and 0.75 of peptide to CaM, two bands were visible on the gel, the fast-moving CaM band and the lower mobility peptide-Ca^{2+}-CaM complex. At a 1:1 molar ratio of peptide to CaM, all of the CaM was gel-shifted, and the intensity of the peptide-Ca^{2+}-CaM band was increased as the result of complex formation (Fig. 3*A*). At still higher molar ratios, no new band was detected on the gel nor did the peptide-CaM complex band change in intensity, indicating a 1:1 binding stoichiometry of peptide to CaM and the absence of multivalent peptide-CaM complexes (Fig. 3*A*). Similar results were obtained when gel shift assays were performed in the presence of 4 M urea. However, in the presence of Ca^{2+} chelator, EGTA, only the pure CaM bands were detected on the gel and no peptide-CaM complex band was visible (Fig. 3*B*), indicating the complex formation is Ca^{2+}-dependent. Peptides from other regions of the Gβ subunit had no effect on the mobility of CaM (data not shown).

Since the synthetic peptide contains a tryptophan residue, whereas CaM contains none, binding of Gβ peptide to CaM was directly studied by monitoring the changes in tryptophan fluorescence (Fig. 4). When the peptide was excited at 295 nm, it exhibited an intrinsic tryptophan fluorescence emission peak

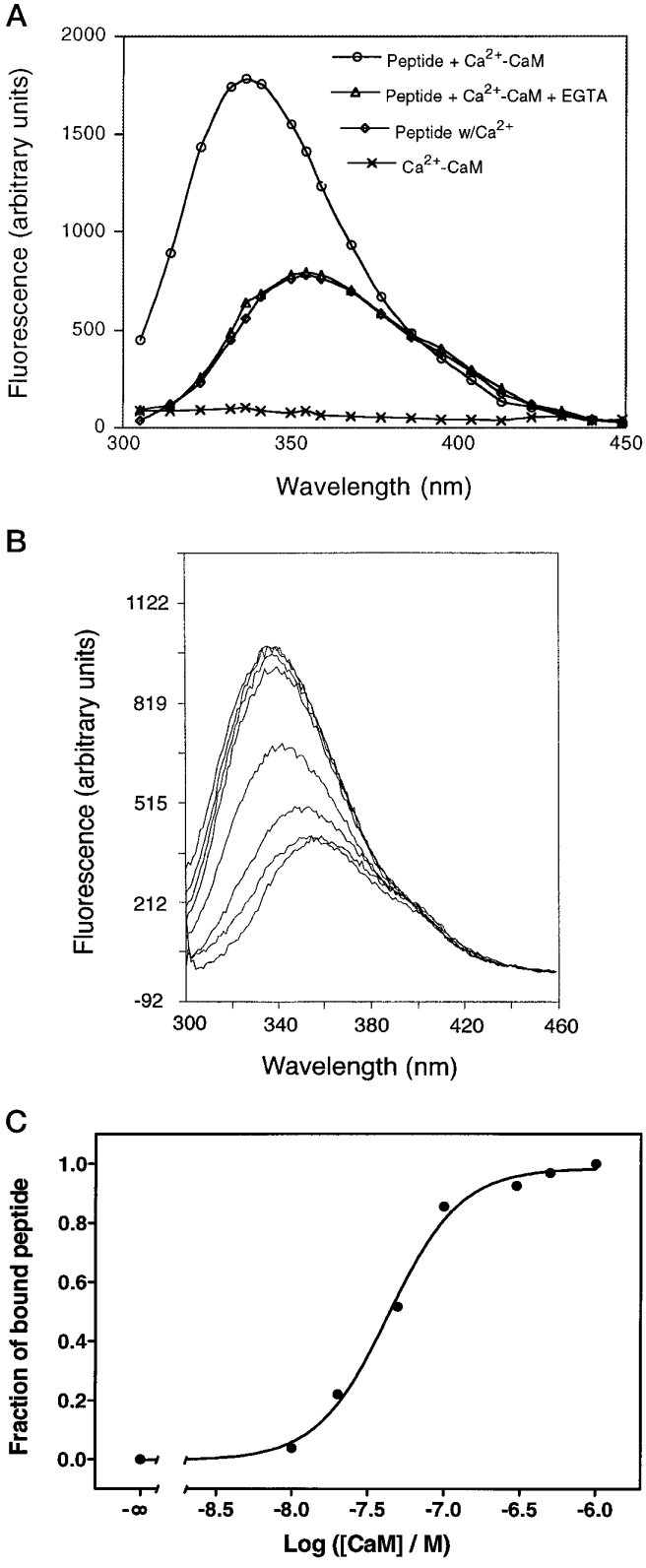

FIG. 4. **Direct interaction of calmodulin with Gβ peptide assayed by measuring the changes in tryptophan fluorescence spectrum.** *A*, Ca^{2+}-dependent interactions of the Gβ peptide with CaM, measured by the emission spectrum of the peptide and peptide-CaM complex. When the peptide (0.1 μM) was excited at 295 nm, the peptide exhibited a fluorescence emission spectrum with a maximal emission peak at 354 nm (*peptide w/Ca^{2+}*). In the presence of Ca^{2+}, addition of CaM not only caused a blue shift of the fluorescence peak but also increased the total fluorescence intensity (*peptide + Ca^{2+}-CaM*). Addition of EGTA completely reversed these fluorescence changes (*peptide + Ca^{2+}-CaM + EGTA*), indicating that the interactions between the Gβ peptide and CaM is Ca^{2+}-dependent. *B*, the Gβ peptide was titrated with different concentrations of CaM in the presence of 0.5 mM $CaCl_2$. *C*, determination of the affinity constant for the interaction between CaM and Gβ peptide. The fraction of bound peptide was calculated and plotted against free CaM concentration. The curve was fitted by a ligand binding equation with a dissociation constant of ~40 nm.

at 353 nm. Addition of CaM in the presence of Ca^{2+} not only caused a blue shift of the fluorescence peak but also increased the maximal fluorescence intensity (Fig. 4*A*). Since Ca^{2+} alone has no effect on the emission spectrum, the observed change must have resulted from the binding of Ca^{2+}-CaM to the peptide. The blue shift in fluorescence emission and the increase in total intensity indicate that the environment of the Gβ peptide became hydrophobic, presumably due to interactions with the hydrophobic domains of CaM upon formation of complex, further confirming the CaM binding property of the peptide. The changes in fluorescence property upon formation of the peptide-CaM complex were fully reversed by the addition of EGTA (Fig. 4*A*), indicating that the binding of CaM to the peptide is Ca^{2+}-dependent. Titration of fixed amounts of peptide with different concentrations of CaM showed a saturation pattern. As shown in Fig. 4*B*, when a 0.1 μM concentration of peptide was used, the fluorescence intensity increased almost linearly with increasing CaM concentrations and reached a plateau at 0.1 μM CaM, confirming a 1:1 tight binding between the peptide and CaM. When the fraction of bound peptide was plotted as a function of free CaM concentration, the dissociation constant (K_d) obtained was ~40 nM (Fig. 4*C*), indicating high affinity binding to Ca^{2+}-CaM. The actual binding affinity could be higher than 40 nM; however, the limitation of this fluorescence measurement does not allow us to test low peptide concentration.

Mutation Analysis of the Putative CaM-binding Domain of Gβ Subunits—To identify the key amino acids essential for CaM binding, two types of mutations were carried out in the putative CaM-binding domain by site-directed mutagenesis. One set contains a number of basic amino acid residues (Arg-42, Arg-48, Arg-49), and the other group involves hydrophobic residues (Ile-43, Leu-51, Leu-55). These specific amino acids were individually replaced by alanine residues. Mutant proteins were then compared with wild type protein for their ability to bind Ca^{2+}-CaM. Both wild type and mutant cDNA were translated *in vitro* together with γ2 subunit in rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine. Fig. 5*A* shows the *in vitro* translated wild type and mutant Gβ proteins separated by SDS-gel and detected by autoradiography. The amount of wild type protein and mutant proteins synthesized are similar under these assay conditions. The *in vitro* translated protein mixtures were incubated with CaM-agarose beads to assess the CaM binding ability. After extensive washing with Ca^{2+}-containing buffer (100–200 bead volume), the bound protein was eluted with EGTA buffer. As shown in Fig. 5*B*, like the wild type Gβ protein, one of the mutants (R42A) retained its ability to bind CaM. However, the binding affinities of other mutants (I43A, L55A, R48A/R49A, and L51A) for CaM were reduced by varying degrees under the same assay conditions. For instance, mutations at residues Arg-48, Arg-49, and Leu-51 dramatically reduced the CaM binding ability of the protein (Fig. 5*B*), suggesting that these residues are critical in the interactions. To further probe the conformation and activity of mutant Gβ subunits, we analyzed the formation of βγ dimers using *in vitro* translated proteins and coimmunoprecipitation with specific anti-$G\gamma_2$ antibodies. The wild type Gβ and its mutant proteins can be precipitated by γ_2 antibodies, and there is little difference in their ability to interact with γ subunits (data not shown), indicating that mu-

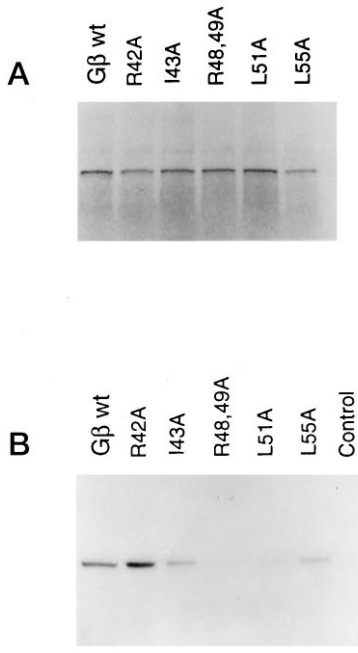

FIG. 5. **Mutation analysis of putative CaM-binding domain of Gβ subunit.** *A*, similar amounts of wild type (*wt*) and mutant $G\beta_1$ proteins were obtained by *in vitro* translation in rabbit reticulocyte lysates (Promega). *B*, CaM binding of *in vitro* translated Gβ protein and its mutants. Compared with wild type Gβ protein, the mutant protein (*R42A*) retained its binding ability to Ca^{2+}-CaM; however, other mutant proteins (*R48,49A*, *L51A*, *I43A*, and *L55A*) showed weak binding ability to Ca^{2+}-CaM, especially mutations at residues Arg-48, Arg-49, and Leu-51.

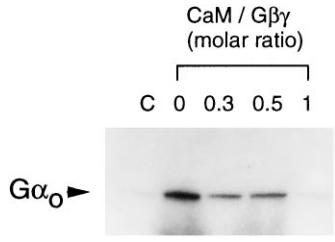

FIG. 6. **CaM inhibited Gβγ-dependent PTX-catalyzed ADP-ribosylation of Gαo.** 0.1 μg of recombinant Gαo was mixed with 0.1 μg of purified bovine brain βγ complex in the presence of indicated molar ratios of CaM. The reaction mixture was incubated for 10 min at room temperature.

tant β subunits could still fold into the native conformation and retain affinity for γ subunits.

Effects of CaM Binding on ADP-ribosylation of Gαo Subunits—To explore the possible physiological functions of CaM binding to Gβγ subunits, we first examined the effects of CaM binding on βγ-dependent pertussis toxin-catalyzed ADP-ribosylation of Gαo subunit. PTX is a bacterial toxin that catalyzes the ribosylation of the C-terminal cysteine residues of Gαo, Gαi, and Gαt subunits. PTX modification blocks the interactions of Gα subunits with receptors and thus blocks the ligand-mediated signal transduction. The labeling of Gαo by PTX requires the Gαoβγ heterotrimer rather than the free Gαo subunit (33). Thus, ADP-ribosylation of αo can be used as a sensitive indicator of formation of αoβγ heterotrimers. Incubation of CaM with bovine brain Gβγ subunits in the presence of 0.1 mM Ca^{2+} inhibited the ribosylation reaction, as shown in Fig. 6. The reduction of labeling is concentration-dependent. At equal molar concentrations of CaM and βγ subunits, the PTX-catalyzed ribosylation of Gαo was almost completely blocked by Ca^{2+}-CaM. Since Ca^{2+} alone had no effect on the reaction, the simplest interpretation of this observation is that Ca^{2+}-CaM formed a complex with Gβγ subunits and this complex lost its ability to interact with Gαo subunit; therefore, CaM inhibited the βγ-dependent ADP-ribosylation of Gαo.

Binding of CaM to Gβγ Has Little Effect on βγ-activated PLC-β2 Activity—To further investigate the nature of CaM binding on Gβγ subunits, we examined its effects on βγ-stimulated PLC-β2 activity. As shown in Fig. 7*A*, CaM has little effect on PLC-β2 activity stimulated by βγ subunits in an *in vitro* reconstituted system. To confirm the observations obtained in the reconstitution experiments, cDNAs encoding PLC-β2, Gβγ, and CaM were transfected into COS-7 cells. Coexpression of PLC-β2 and βγ subunits increased inositol 1,4,5-trisphosphate release 3–5 fold (Fig. 7*B*, *column 3*). However, cotransfection of CaM expression plasmids had little effect on the βγ-stimulated PLC-β2 activity (Fig. 7*B*, *column 4*). These results indicated that in the Gβ subunit, the domain responsible for PLC-β2 activation is distinct from the domain binding to CaM, suggesting that different domains of the β subunit are involved in interactions with different effector proteins.

Inhibition of Ca^{2+}-CaM-dependent Enzyme, CaM Kinase II, by Gβγ Subunits and Its Peptide—The putative CaM-binding domain of Gβ subunits was further characterized by using the synthetic peptide derived from Gβ to inhibit Ca^{2+}-CaM-dependent activation of CaM kinase II. Concentration-dependent effects of the peptide on CaM kinase II activity were examined. As shown in Fig. 8*A*, the peptide was a potent inhibitor of CaM kinase II activity. At a 1:1 binding ratio of Gβ peptide to CaM, the peptide totally inhibited the Ca^{2+}-CaM-dependent activation of CaM kinase II, suggesting that the binding affinity of Gβ peptide is comparable to the affinity of CaM kinase II toward Ca^{2+}-CaM and should be in the nM range. Other peptides from the N-terminal regions had no effect on the Ca^{2+}-CaM-stimulated enzymatic activity (data not shown). To examine whether Gβγ subunits can also inhibit the Ca^{2+}-CaM-dependent CaM kinase II activity, Ca^{2+}-CaM was incubated with brain Gβγ subunits for 30 min at room temperature. Then the Ca^{2+}-CaM-stimulated CaM kinase II activity was assayed. Fig. 8*B* shows that brain Gβγ subunits inhibited 70–80% of Ca^{2+}-CaM-stimulated CaM kinase II activity, indicating that βγ can competitively bind to Ca^{2+}-CaM.

DISCUSSION

Previous studies have demonstrated that G protein subunits can act as potent inhibitors of the Ca^{2+}-CaM-stimulated phosphodiesterase activity (23, 24), probably through interactions of Gβγ with CaM. In this report, we show direct binding of Gβγ subunits with CaM. This CaM-binding property of Gβ is Ca^{2+}-dependent and conserved in known Gβ subunits, including the most diverse β5 subunit. We also identified and characterized the CaM-binding domain in βγ subunits using three different methods. In the gel mobility shifting assays and tryptophan fluorescence assay, a conserved 25-amino acid peptide in the N-terminal region of Gβ subunits was found to interact with CaM. By modifying specific amino acids in this region, we identified some key residues (Arg-48, Arg-49, Leu-51, Ile-43, Leu-55) that play an important role in the binding of βγ to CaM.

The interaction between Gβγ subunits and CaM is Ca^{2+}-dependent. Ca^{2+} is an important intracellular messenger in many cellular functions including cell growth and development (34, 35). Many seven-transmembrane receptors activate the G protein subunits, which in turn activate PLC-β, resulting in the production of inositol 1,4,5-trisphosphate and diacyglycerol from $PtdInsP_2$. Inositol 1,4,5-trisphosphate acts as an intracellular second messenger by binding to the inositol 1,4,5-trisphosphate receptors in the endoplasmic reticular mem-

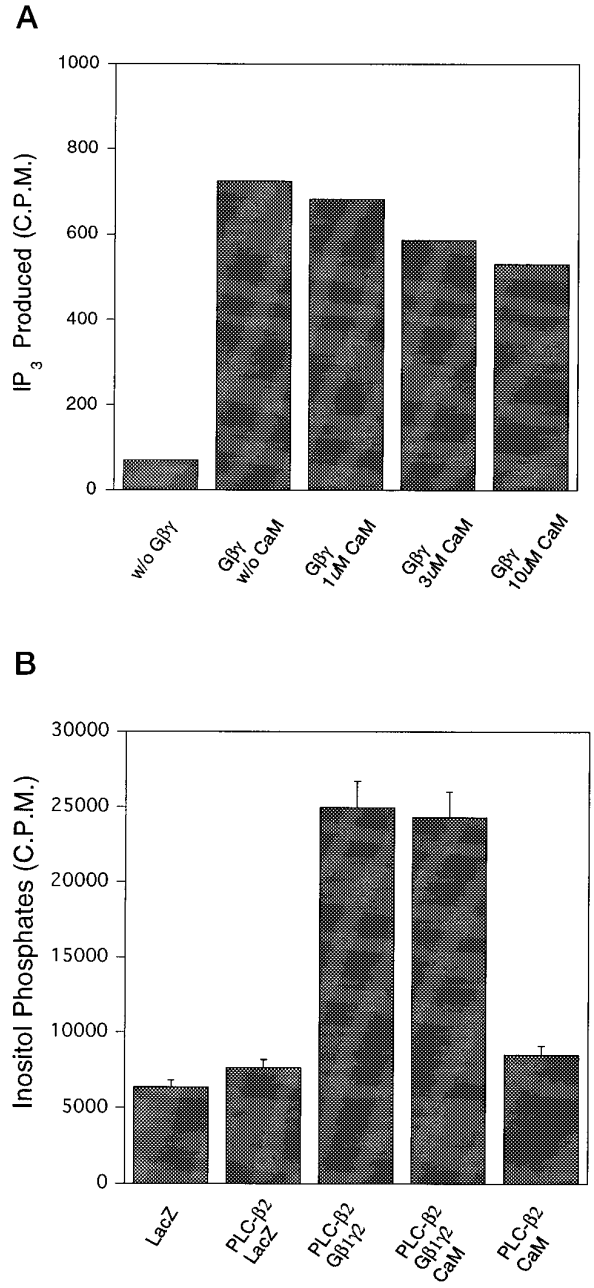

FIG. 7. **Calmodulin has little effect on Gβγ-stimulated PLC-β2 activity.** *A*, *in vitro* reconstitution assay of PLC-β2 activity. The Gβγ-activated PLC-β2 activity was assayed in a reaction mixture containing PLC-β2, brain or retinal Gβγ, 50 μM $CaCl_2$, 10 mM LiCl, phospholipid vesicles, and different concentrations of CaM. The released inositol 1,4,5-trisphosphate (IP_3) was extracted, separated, and subjected to scintillation counting. *B*, *in vivo* transfection assays of PLC-β2 activity. Accumulation of inositol phosphates was measured in COS-7 cells transfected with expression vectors carrying cDNAs corresponding to β-galactosidase (*lacZ*), PLC-β2, $Gβ_1$, $Gγ_2$, and CaM. The cDNAs transfected are indicated *beneath* each column.

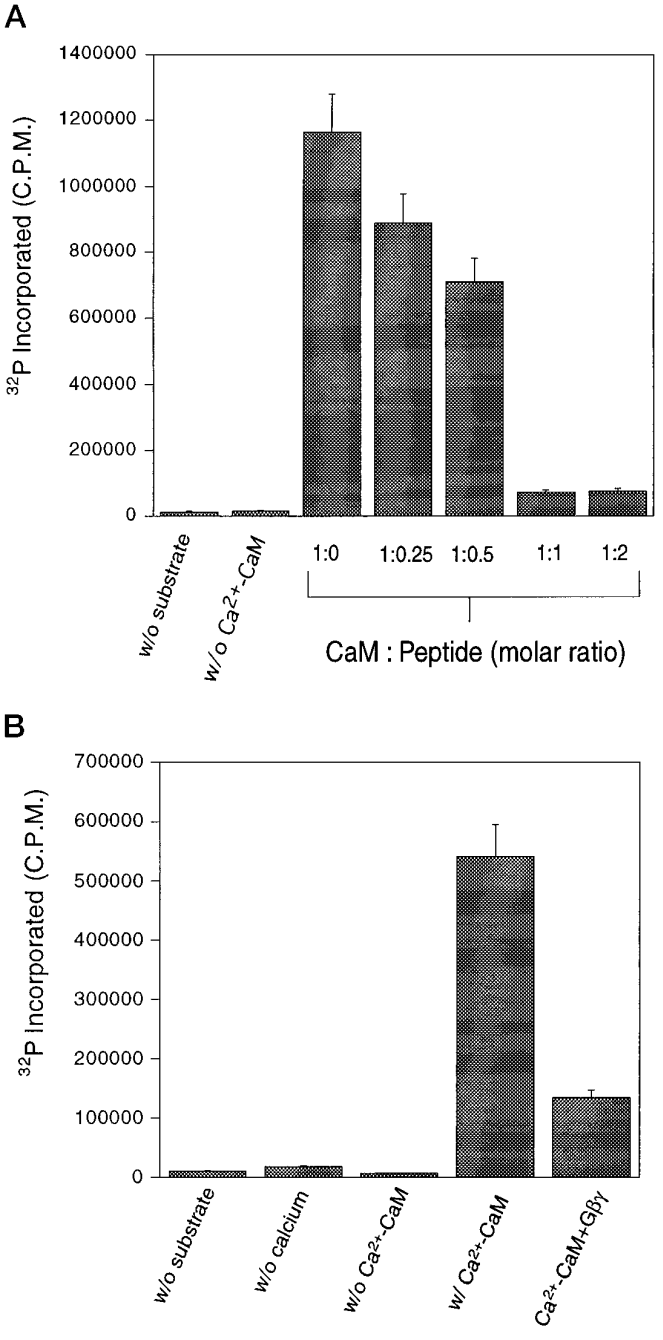

FIG. 8. **Inhibition of Ca^{2+}-CaM stimulated CaM kinase II activity by Gβγ and its CaM-binding peptide.** *A*, the putative CaM-binding peptides derived from Gβ inhibited the Ca^{2+}-CaM-dependent CaM kinase II activity. Different molar ratios of Gβ peptide were incubated with CaM, then added to the kinase assay mixture. The final concentration of CaM was kept the same in all the reactions. *B*, inhibitory effects of brain Gβγ on CaM kinase II activity. At equal molar ratios, Gβγ can compete for CaM, inhibiting the Ca^{2+}-CaM-dependent CaM kinase II activity.

brane, triggering the release of Ca^{2+} from the endoplasmic reticulum and therefore increasing the intracellular Ca^{2+} concentration. Ca^{2+} is also believed to organize and stabilize CaM domain structure in a conformational state that can bind target proteins (32, 36). The calmodulin concentration in some cells, *e.g.* neuronal cells, is in the order of micromolar. Calcium release can thus convert 10–50% of these molecules to the Ca^{2+}-bound form. The affinity of Gβγ for Ca^{2+}-CaM is sufficiently high so that under these conditions much of the free βγ in the cells should be in the CaM-bound form. Therefore, an increase in intracellular Ca^{2+} concentration could selectively regulate the interactions of Gβγ with a number of other proteins through binding with CaM.

The interaction of Gβγ with Ca^{2+}-CaM could play an important role in the cross-talk mechanism between different G protein pathways (37). Ca^{2+}-CaM has been shown to regulate the formation and hydrolysis of cAMP. In the Gαs-coupled pathway, both adenylyl cyclases and phosphodiesterases are Ca^{2+}-CaM-dependent, which could serve as the convergence point for Ca^{2+}-dependent and Gαs-dependent stimuli.

The primary structure of the identified CaM-binding domain of G$\beta\gamma$ shows features similar to some other CaM-binding proteins and inhibitors (21, 28, 38–45). For instance, the identified β CaM-binding domain contains a high percentage of hydrophobic residues and an excess of positively charged residues, a property found in all CaM-binding peptides. It is believed that the basic residues contribute to CaM binding via electrostatic interactions with acidic residues in CaM, whereas the hydrophobic amino acids seem to play a more important role in CaM binding through interactions with the hydrophobic patches of the globular domains of CaM (38, 39). From x-ray structural analysis, 80% of all contacts are van der Waals interactions, and the interaction appears to involve two key hydrophobic amino acids separated by eight residues, although flexibility does exist in the interaction because of the flexible central helix of CaM (36). CaM contains two globular domains, and these two domains are connected by a long alpha helical segment (32, 36). When different CaM binding targets are recognized, this long central segment allows different relative positioning of the two lobes of CaM. Therefore, although the secondary structure of the Gβ CaM-binding domain is not a typical amphipathic α helix (46), CaM could still position itself to bind to the Gβ protein, possibly by stabilizing a change in the conformation of the N-terminal domain of Gβ protein. On the other hand, a number of CaM-binding proteins have been identified and shown to contain sites that are not typical amphipathic helices but consist of basic amino acids interspersed with nonpolar amino acids (52–54).

The binding of CaM to G$\beta\gamma$ interfered with the formation of G$\alpha\beta\gamma$ trimers as assayed by the inhibition of PTX-catalyzed ADP-ribosylation of Gαo. Based on the secondary structure of the β subunit, the N-terminal region of the β subunit is in close proximity to the N-terminal portion of Gα subunit (47–49). It has been reported that effector activation by the $\beta\gamma$ subunits is blocked upon the addition of the Gα subunit presumably by heterotrimerization (1, 13). Thus, by interacting with the N-terminal domain of β subunit, the Ca^{2+}-CaM complex could affect the heterotrimer formation of G$\alpha\beta\gamma$.

Interaction of CaM with $\beta\gamma$ has little effect on the G protein $\beta\gamma$ subunit-activated PLC-β2 activity. These results support the notion that interaction of different $\beta\gamma$-responsive effectors is mediated by distinct domains of G$\beta\gamma$ (50, 51). By using a series of chimeras between *Dictyostelium* and mammalian β subunits, a small C-terminal segment of Gβ was identified as responsible for the activation of PLC-β2 (50). The CaM-binding domain of Gβ identified in this report is located in the N-terminal region. This region of Gβ was suggested to play a role in the interaction of adenylyl cyclase type 2, the muscarinic receptor-gated atrial inwardly rectifying potassium channel (GIRK1), and in the activation of mitogen-activated protein kinase pathways (50, 51). It will be of great interest to determine whether binding of CaM to G$\beta\gamma$ can affect the activation of the potassium channels (GIRK) and mitogen-activated protein kinase pathways.

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